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MIPR: 93MM3548

TITLE: Evaluation of Metabolic Fate of Munitions Material (TNT & RDX) in Plant Systems and Initial Assessment of DNA Mutation Spectra as a Biomarker

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REPORT DATE: June 1995

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 1995	Final (8 Feb 93 - 31 May 95)

4. TITLE AND SUBTITLE
Evaluation of Metabolic Fate of Munitions Material (TNT & RDX) in Plant Systems and Initial Assessment of DNA Mutation Spectra as a Biomarker

5. FUNDING NUMBERS
MIPR 93MM3548

6. AUTHOR(S)
Dominick A. Cataldo, Frederick Leung, Robert J. Fellows, Ann E. Jarrell, Scott D. Harvey

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Battelle Memorial Institute
Richland, WA 99352

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command,
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release;
distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Munitions material can enter the environment as a result of manufacturing activities and field usage. Predictor methodologies, or biomarkers would enhance evaluation of environmental impacts. The goal of this exploratory study was to assess deoxyribonucleic acid (DNA) mutation frequency as a biomarker for munitions exposure. The approach involved, 1) the resolution of an effective repetitive sequence probe for the identification of characteristic mutations, and 2) the development of a testing media [a clonal cell line of carrot (*Daucus carota*) suspension cells]. Commercially available probes demonstrated marginal resolution therefore a low-C_0t library was then constructed. Three colonies from the low-C_0t DNA library were screened and the DNA isolates sequenced. A suspension culture of carrot (*Daucus carota*) was developed. A mutation spectra experiment was initiated at a 10-mg TNT/L exposure concentration with the attempt to clone over 1500 single TNT-exposed cells. Over the following six months greater than 98% of the initially isolated cells were unable to survive and produce micro calluses. The remaining calli were too few to be statistically significant and the experiment was terminated. The biomarker concept itself remains to be disproved, but the need for large numbers of uniform clones to differentiate true mutations suggest that more direct techniques using whole tissues need to be developed.

14. SUBJECT TERMS
Munitions, Material, DNA

15. NUMBER OF PAGES
26

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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*U.S.GPO:1993-0-358-779

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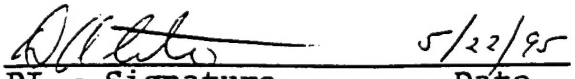

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EXECUTIVE SUMMARY

Munitions material such as 2,4,6-trinitrotoluene (TNT) and its combustion and decomposition products can enter the environment as a result of manufacturing activities as well as field usage and disposal. In assessing the potential environmental risk of these residues, the mere presence of specific munitions-related products in the environment cannot be indicative of the presence or severity of an impact. Instead, characteristic deleterious effects (i.e., chronic damage patterns) of these products within the biotic components of terrestrial and aquatic environs must be considered. For terrestrial systems, a critical link is accumulation by green plants, the fundamental component of the terrestrial food chain. Any evaluation of environmental risk to green plants would be decidedly enhanced through the application of predictor methodologies, or biomarkers, to identify actual characteristic impacts from munitions before a problem becomes technically, or politically, difficult to resolve.

The goal of this study was to explore deoxyribonucleic acid (DNA) mutation frequency as a biomarker for munitions exposure. The objective was to demonstrate that unique, reproducible genetic mutation spectra can be produced in response to TNT contamination. This study was exploratory in nature, and designed to assess the efficacy of the repetitive sequence-DNA fingerprinting (low-C_0t) technique to identify characteristic TNT-induced mutations. The approach involved a two-pronged effort for 1) the resolution of an effective repetitive sequence probe for the identification of characteristic mutations, and 2) the development of a testing media [a clonal cell line of carrot (*Daucus carota*) suspension cells].

DNA Repetitive Sequence Analysis

Modification of established techniques for the isolation of higher plant DNA from the carrot tissue maximized DNA yield to greater than 80 to 100 μg/g fresh weight tissue. A pooled supply of carrot DNA was then prepared and subsampled. The subsamples were then processed with differing restriction enzymes and the DNA fragments separated through agarose gel electrophoresis and blotted into a nylon membrane. Initial probing with a commercially available nonradioactive $(CAC)_5$ probe demonstrated marginal resolution and indicated that this was not the optimum probe for DNA fingerprinting analysis.

A low-C_0t library was then constructed to select more effective probes. The genomic DNA was sheared and allowed to reanneal to a C_0t value of 1. Double-stranded DNA was isolated, blunt ended, the gaps were filled in with klenow fragment, and the repaired, double-stranded

DNA subcloned into the Sma I site of a pUC18 vector and used to transform *E. coli* to generate the library.

Three colonies from the low-C_0t DNA library were screened and the DNA inserts sequenced. When these inserts were used as probes, distinct DNA profiles were obtained from carrot genomic DNA cut with the various restriction endonucleases. The numbers of DNA bands per lane ranged from two to eight depending on the enzyme used.

Establishment of Carrot Cell Cultures

As a uniform source of plant material, a suspension culture of carrot (*Daucus carota*) was initiated using the techniques described in Dixon (1985). This basically consisted of establishing isolated carrot root callus cultures grown on solid Murashige and Skoog medium. The callus cultures were maintained through a minimum of two transfers (~3 months).

Assessment of Relative TNT Toxicity to Carrot Cell Cultures

To determine the TNT concentration that would ensure a stress but not prove too toxic to kill the cells, the rates of isolated cell respiration were followed for 72 h following a 1-h exposure to TNT at concentrations ranging from 0 to 100 mg/L. The results showed that under our growth conditions, an exposure concentration of 10 mg/L was optimal because the cultures exhibited an initial decline in respiration rate but recovered and exhibited growth within 72 h while higher concentrations proved lethal.

Mutational Spectrum Study

The criteria required for a biomarker are 1) that the change in a living organism induced by the stressor be stable, and 2) the change be characteristic for a particular stressor. Statistical resolution of a biomarker requires large sampling numbers to distinguish a true biomarker from spontaneous or non related mutation. Cell cultures can be cycled to provide a large population of individual cells of which the majority are statistically at the same developmental stage. Further in a liquid suspension, all of the cells should receive a similar dosage. Unfortunately, even though the suspension culture is homogeneous, resolution of specific genetic changes may be lost in the background noise of the large population. Thus, examining individual cell "subclones" or populations derived from single isolated exposed cells was essential. To do this, >1500 individual cells from each treatment would have to be cultured. This number was chosen to provide a final sample number of at least 100 to 400 separate cloned populations for DNA analysis and was projected based on the assumption of an optimal 5 to 25% survival rate.

Individual cells were exposed to 10 mg/L TNT and then isolated and cultured. Over the following 6 months, greater than 98% of the initially isolated cells were unable to survive and produce microcalluses. The remaining calli were too few to be statistically significant and the experiment was, therefore, terminated.

The loss of these cell cultures meant that the statistical significance required to demonstrate unique mutational spectra was not achieved within the time frame of the project. The concept itself remains to be disproved, but the highly risky nature of the experimental approach and the need for large numbers of uniform clones to differentiate true mutations suggest that more direct techniques using whole tissues need to be developed. These latter may prove more cost effective and amenable to field validation.

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1.0 INTRODUCTION

Munitions material such as 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and their combustion and decomposition products can enter the environment from production and manufacturing activities and field usage and disposal (Small and Rosenblatt 1974; Spanggord et al. 1983; Ryon et al. 1984). In assessing the potential environmental risk of these residues, the mere presence of specific munitions-related products in the environment cannot be indicative of the presence or severity of an impact. Both the relative accessibility to subsequent dependent components in the food chain (e.g., partitioning to commonly edible tissues) as well as characteristic deleterious effects (i.e., chronic damage patterns) of these products within the biotic components of terrestrial and aquatic environs must be considered. For terrestrial systems, a critical link is accumulation by green plants, the fundamental component of the terrestrial food chain.

Much of the research performed to date has addressed questions of controlling processes and observed impacts. These have served to establish the extent of potential risk to the environment. Any subsequent direct evaluation of environmental risk as required through regulatory processes must include these evaluations and other recognized evidence of adverse impacts. The application of early predictor methodologies (called biomarkers) to identify actual characteristic impacts would more effectively identify adverse environmental changes before a problem becomes technically, or politically, difficult to resolve.

The goal of the effort described in this report was to explore deoxyribonucleic acid (DNA) mutation frequency as a biomarker. The objective was to demonstrate that unique, reproducible genetic mutation spectra can be produced in response to exposure to TNT. This study was exploratory in nature and designed to assess the efficacy of the repetitive sequence-DNA fingerprinting (low-$C_o t$) technique to identify characteristic TNT-induced mutations. This would aid in the evaluation of potential impacts of munitions materials residing within specific environs.

1.1 REVIEW OF RELATED LITERATURE

Several studies have been conducted on the development of analytical methods and subsequent characterization of munition parent compounds and decomposition products associated with waste streams, impoundments, and/or releases from production sites. However,

few have dealt with the environmental persistence, bioavailability, and metabolic detoxification of these materials. Even fewer have addressed impacts of these compounds on the genetic makeup of the surrounding environment.

Different chemicals have been demonstrated to cause characteristic mutational spectra in viral DNA (Benzer and Freese 1958). Since then, mutational spectra studies have been investigated using clone-by-clone techniques in bacteria, rodent cells, vector-carried genes replicating in human cells, and human fibroblasts' endogenous HPRT gene (Eisenstadt et al. 1982; Roilides et al. 1988; Yang et al. 1987; Chen et al. 1990). The possibility therefore exists that munitions materials may themselves be capable of producing characteristic mutations within the DNA of higher plants. These distinct mutations (mutational spectra) may prove to be another unique indication of the presence of the material in the plant tissue, and thus become a biomarker. The reasoning is that mutational spectra can be highly specific to a given mutagen. One of the impediments to this approach has been the difficulty of analyzing a large set of separate mutants for quantitative and statistically precise comparison among mutational spectra.

Of the approaches available to assess this possibility, one was selected overall. This approach employs DNA fingerprinting using repetitive sequences and would aid in identifying polymorphic DNA fragments altered by exposure to TNT. DNA fingerprint assays have previously been used to determine characteristic genomic rearrangements in human and mouse tumors (Thein et al. 1987; Armour et al. 1989; Boltz et al. 1990; Inoue et al. 1992; Matsumura and Tarin 1992). Ledwith et al. (1990) examined genomic rearrangements in spontaneous and DMBA-induced liver tumors in CD-1 mice using DNA fingerprint analysis with repetitive sequences as probes. The study discovered many more rearranged bands in DMBA-induced liver tumors than in spontaneous liver tumors. Honma et al. (1994) and others have observed that the mutation spectrum determined by DNA fingerprinting assay is probe dependent.

Certain probes have the potential to detect more mutations than others. In 1994, Honma et al. used minisatellite DNA probes in a DNA fingerprinting assay as a mutation assay system to detect recombination mutations in cultured human cells. The system was used to monitor DNA rearrangements in 3T3 cells transformed spontaneously or by treatment with 3-methylcholanthrene (MCA), UV-C, and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) in combination with MCA. They observed that the genomic DNA in 3T3 cells is unstable and susceptible to rearrangement. Further, the DNA's instability is elevated during transformation. MCA and UV-C induced rearrangements and TPA enhanced the effect of MCA when treated in

combination. This approach was limited by the requirement of a large number of cells which carry the same type of mutation in order to reach a detection threshold and may therefore be statistically difficult to detect in a small population.

This application of minisattellite DNA probes in a DNA fingerprinting assay technique was similar to that employed by Leung et al. (1992) to determine characteristic banding patterns of taxonomically different fungal species. The hope was that similar slightly modified procedures could be used to determine the extent to which munitions impact the plant genome. Thus mutation frequency could readily be employed as a biomarker and provide both early detection of change and diagnostic information as to the type of contaminant.

1.2 TECHNICAL APPROACH

The mutagenic spectrum studies employed TNT as the contaminant. The studies included repetitive sequence-DNA fingerprinting technique development and refinement of *in vitro* analysis experimental procedures using isolated cell preparations. To ensure a source of uniform higher plant genetic material capable of responding to a TNT insult, a suspension cell culture of carrot (*Daucus carota*) was established.

2.0 MATERIALS AND METHODS

This study was exploratory in nature, and designed to determine whether higher plant DNA mutation frequency could be used as a biomarker with respect to TNT and its transformation products. The approach involved a two-pronged effort for both the resolution of an effective repetitive sequence probe for the identification of characteristic mutations, and the development of a testing media [a clonal cell line of carrrot (*Daucus carota*) suspension cells].

2.1 DEVELOPMENT OF A REPETITIVE SEQUENCE ASSAY

The experimental steps in developing a repetitive sequence assay for DNA mutation evaluation involve 1) extraction of target plant DNA and digestion with enzymes, 2) isolation of repetitive sequences, and 3) fractionation and characterization of the repetitive sequences.

2.1.1 DNA Extraction and Isolation of Repetitive Sequences

This approach was an attempt to isolate the gene sequence(s) affected by the mutagen. It was anticipated that from this, a specific probe might be developed and used to quantify the impact of TNT with respect to DNA mutation.

A modified procedure described by Rogers and Bendich (1988) was employed to extract high molecular weight genomic DNA from carrots. The extraction procedure included freezing of a 10-g (fresh wt.) sample of carrot cells in a 50-mL polypropylene centrifuge tube with liquid nitrogen after which the material was lyophilized and subsequently ground to a fine power under liquid nitrogen in a mortar and pestle. The powder was then transferred to an Oak Ridge tube and three to five volumes of hot (65°C) 1X cetyltrimethylammonium bromide (CTAB) buffer [2% CTAB (w/v) (Sigma Chemical Co., St. Louis, MO), 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% (v/v) β-mercaptoethanol] added. The tube was then placed into a 65°C water bath for 15 min. Next, one volume of chloroform/isoamyl alcohol (24:1) was added to the tube and the contents thoroughly mixed by hand until an emulsion formed. The tube was then centrifuged at 8500 x g at 4°C for 5 min. The supernatant was then transferred to a new tube. The original pellet was again extracted with a chloroform/isoamyl alcohol mixture, centrifuged, and the supernate combined with the first. A one volume amount of a 1.0% (w/v) CTAB precipitation buffer [1% CTAB (w/v), 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% (v/v) β-mercaptoethanol] was then added to the supernate. The contents were then mixed and centrifuged again at 8500 x g for 5 min. The supernate was decanted and the resulting final pellet rehydrated in high-salt TE buffer

(10 mM Tris-HCl, pH 8.0,1 mM EDTA, 1.25 M NaCl). Two volumes of cold 100% ethanol were then added and the tube held at -20°C for 15 min to 30 min. The tube was then centrifuged again at 8500 x g at 4°C for 5 min and the resulting pellet washed with 70% (v/v) ethanol. The DNA pellet was then air dried and rehydrated with 0.1 X TE (10 mM Tris-HCl, pH 8.0,1 mM EDTA, 1.25 M NaCl). The anticipated yield should be in the range of 50 µg DNA per 1 g fresh weight of tissue.

2.1.2 Generation of Low-C_ot DNA Library

Approximately 200 µg of the genomic DNA was sheared to lengths between 300 bp and 2 Kb using an ultrasonicator. The samples were maintained at 4°C during the shearing with the aid of an ice-water bath. Sheared DNA fragments were denatured by boiling the material for 5 min at 100°C and subsequently allowed to reanneal in a 60°C water bath to a C_ot value of 1. Double-stranded DNA fragments were separated from the single-stranded DNA by passing the mixture through a 5-mL water-jacketed hydroxyapatite (Bio-Gel HTP, BIO•RAD, Richmond, CA) packed (0.5 g) column. The hydroxyapatite had previously been equilibrated with 0.12 M sodium phosphate buffer (pH 6.8). The material containing the double stranded fraction was then eluted from the column with four 2.5-mL alliquots of the 0.12 M sodium phosphate buffer (pH 6.8). Double-stranded DNA was blunt ended, gaps were filled in with klenow fragment, and then the DNA was subcloned into the Sma I site of a pUC18 vector and transformed into *E. coli* .

2.1.3 Generation of DNA Probes and DNA Fingerprinting

Three of the resulting *E. coli* clones containing inserts were subsequently isolated and cultured. Miniprep DNA was obtained from these carrot low-C_ot DNA library colonies and were subsequently subjected to sequence analysis in both orientations by the dideoxy chain termination method (Sanger et al. 1980). The membranes were initially probed with a commercially available ^{32}P-labeled $(CAC)_5$ probe. Additional ^{32}P-labeled DNA probes were generated by performing polymerase chain reaction (PCR) using M13 primers in the presence of ^{32}P-dATP and using the miniprep DNA as templates. The PCR products were boiled and snap-cooled before use.

For the DNA fingerprinting assay, 20 µg each of carrot genomic DNA was subjected to restriction digestion by Alu I, Sau 3aI, Hae III, Eco RI, and Hind III. The digestions were conducted according to the manufacturer's recommendation. The digested DNA samples were electrophorectically separated through a 0.7% w/v agarose gel, transferred onto an uncharged nylon membrane, probed with a ^{32}P-labeled low-C_ot DNA insert probe at 64°C overnight, and

processed for autoradiography at -80°C with an intensifying screen.

2.2 ESTABLISHMENT OF CARROT CELL CULTURES

To provide a constant source of plant material of uniform genetic characteristics for the DNA experiments which would be relevant to the whole plant efforts, a suspension culture of carrot (*Daucus carota*) was initiated using the techniques described in Dixon (1985). Establishing the suspension culture required the initial generation of isolated carrot root callus cultures on solid Murashige and Skoog medium (Sigma, St. Louis, MO). To accomplish this, the tissue was aseptically cut from the cortical region of the root and transferred to 50-mL culture vials containing the filter sterilized media. The vials were incubated at 25°C in the dark for 3 weeks, at which time calluses formed from the tissue were subsampled and transferred to new vials containing the same media. Two additional transfers were made in the same manner.

At this time, 0.5-cm-diameter fragments of the callus were aseptically transferred to a 2000-mL flask containing 500 mL of liquid Murashige and Skoog medium (minus the 2% agar of the solid media). The flasks were wrapped in foil to exclude light and incubated on a rotary shaker bath (60 rpm) at 25°C for an additional 2 weeks. The flasks were then removed from the shaker, the large clumps of tissue allowed to settle for 10 min, and a 100-mL aliquot of suspended single cells removed from the flask and transferred aseptically into flasks containing fresh media. The new flasks were then returned to the shaker for two more weeks of incubation. Two more transfers were made in this manner before a constant cell line was established. Descriptions of the preliminary range-finding experiments performed with the suspension cell cultures are given in Section 3.

3.0 RESULTS AND DISCUSSION

This study involved resolving an effective repetitive sequence probe for the identification of characteristic mutations (Section 3.1), as well as developing a testing media (a clonal cell line of carrrot suspension cells) (Section 3.2). These two efforts were then combined to assess the efficacy of the technique.

3.1 DNA REPETITIVE SEQUENCE ANALYSIS

This initial task was pursued employing standard molecular biological techniques described in Section 2.

3.1.1 DNA Extraction

Initial experiments were conducted using fresh-frozen intact, carrot roots as well as the isolated cells from the suspension cell culture. The DNA yields from these attempts were relatively low (<10 to 20 μg DNA/g tissue), and the procedure was modified to include a freeze-drying or lyophilization step. This increased the yield at least four-fold for each subsequent extraction. For the inital characterization, five extractions were carried out over a 4-week period and the DNA pooled for subsequent analysis. A representative spectrophotometer printout of a carrot DNA genomic DNA preparation is given in Figure 3.1.

Subsamples of the pooled carrot genomic DNA (15 μg) were each cut with either Alu I, BamH I, EcoR I, Hae III, Hind III, Hinf I, or Sau 3aI restriction enzymes. After the digestion, DNA fragments were separated through agarose gel electrophoresis and transferred into a nylon membrane. The membrane was initially probed with a commercially available radioactive ^{32}P-$(CAC)_5$ probe. Figure 3.2. shows the autoradiogram of the carrot DNAs cut with various enzymes and probed with the ^{32}P-$(CAC)_5$ probe. The autoradiogram demonstrates that the quality of the carrot genomic DNA is good for DNA fingerprinting. Even though the ^{32}P-$(CAC)_5$ probe gives multiple banding patterns in the DNA profile, the high background made it less than an optimum probe for DNA fingerprinting analysis. Therefore, a low-C_0t library was constructed to select more effective probes.

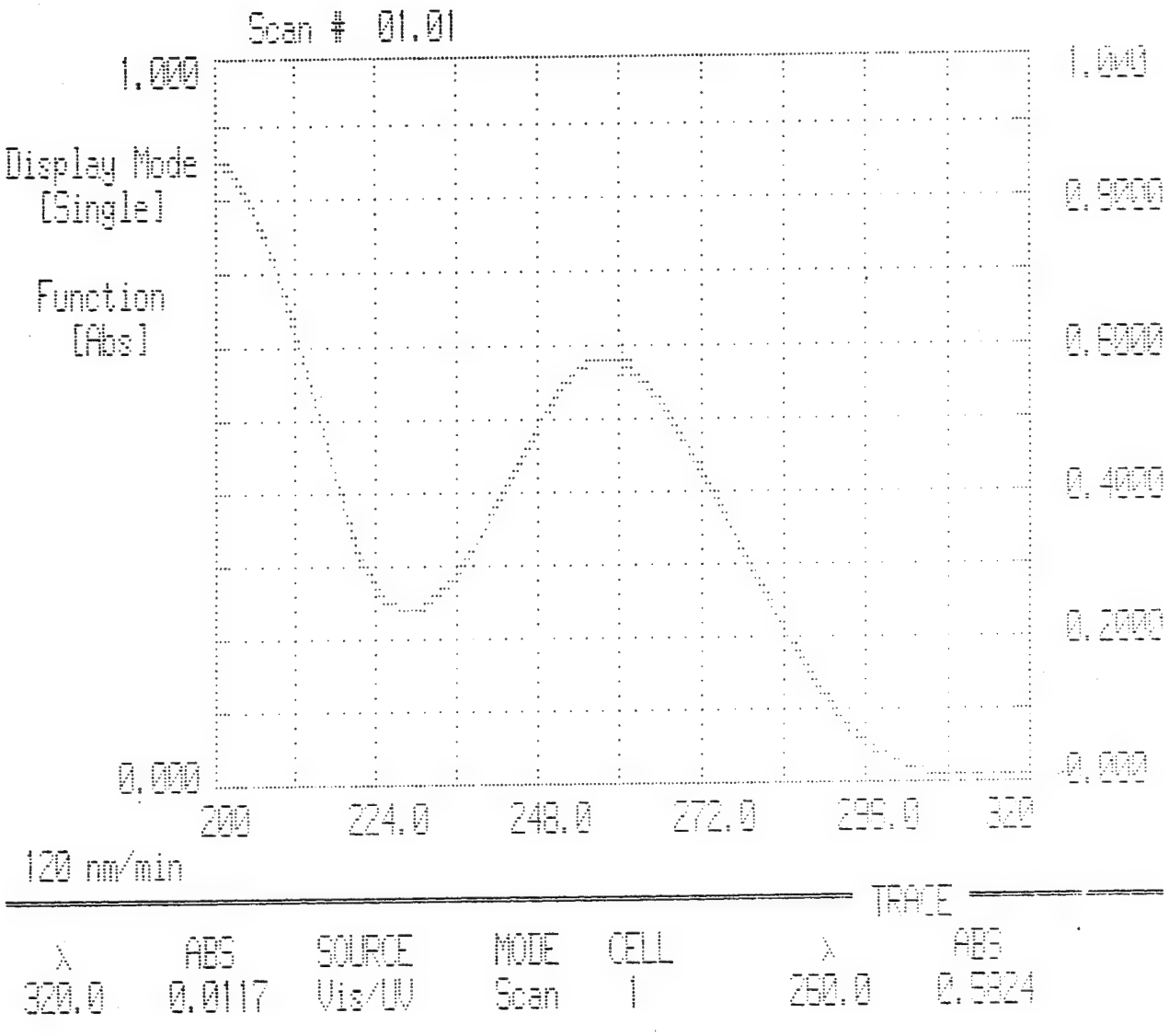

FIGURE 3.1. Spectrophotometer Printout of a Carrot DNA Genomic DNA Preparation. The yield of that particular preparation is 58 µg/g and the 260/280 ratio is 2.08.

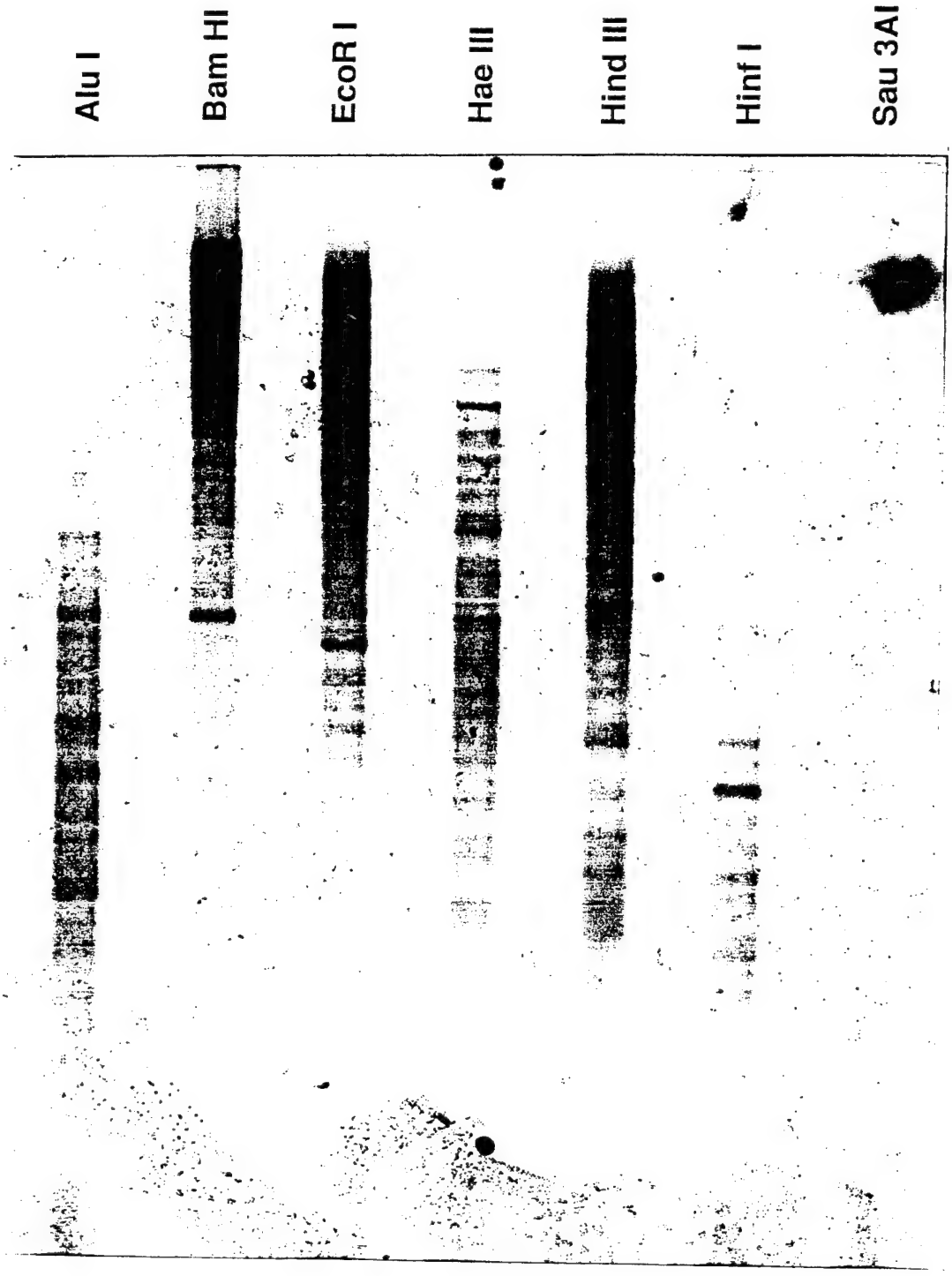

FIGURE 3.2. Autoradiogram of the Carrot DNAs Cut with Either Alu I, BamH I, EcoR I, Hae III, Hind III, Hinf I, and Sau 3aI Restriction Enzymes and Probed with the $(CAC)_5$ Probe

3.1.2 Generation of DNA Probes from the Low-C_0t DNA Library

Three colonies from the low-C_0t DNA library were screened and the DNA inserts sequenced (Tables 3.1, 3.2, and 3.3). When we employed these as PCR-generated ^{32}P-labeled probes in a fingerprint assay, distinct DNA profiles were obtained from carrot genomic DNA cut with various enzymes. The number of DNA bands per lane ranged from two to eight depending on the enzyme used, with SAU 3aI and Alu I providing the greatest resolution.

TABLE 3.1. Insert Sequence from Carrot Colony No. 1. Sequence range: 1 to 142.

```
                    *                   *                   *                   *
GGA CTC AAA ATT TGA ACG AAA TTT GGG TAC CCT AGG AGA ACC CCC CAG
50
 *                   *                   *                   *
GAT TTA ATC CTT TAC CTC TCA ATG ACA TAT CGA GAA AAA GGA CCG GCC
     100
     *                   *                   *                   *
CAA GTC CCT GGA AGG GTC GCC AGA GAG GGT GAG AGC CCG TCG TCG TGC C
```

TABLE 3.2. Insert Sequence from Carrot Colony No. 5. Sequence range: 1 to 50.

```
                                                                      50
                    *                   *                   *       *
GCT GCG CTC GTT GGG GCC TCC TCG GGC TCT TCA GGC GTC CTG GGG CAC GC
```

TABLE 3.3. Insert Sequence from Carrot Colony No. 16. Sequence range: 1 to 140.

```
                    *                   *                   *                   *
GAA TAA GGA TGG AGA GAT GTG TGT TAA CCA GCC AAG CCA CTC TGC AGT
50
 *                   *                   *                   *                   *
CTC CCA AAA GAC TGT AGA TGT TAA TAT GGA ATT AAA CGC ATC CCT AGC
     100
     *                   *                   *                   *                   *
AGC ATC CTC CCA AAA GGA TGT TGC TAT TGA AAA TAG TCC TCA AC
```

3.2 ESTABLISHMENT OF CARROT CELL CULTURES AND TOXICITY SCREENING

A suspension cell culture line of carrot cells was established using the standard techniques described in Dixon (1985). This culture was carried through six separate transfers before use in the toxicity screening experiments described below.

It was important to select a concentration of TNT that would ensure that a stress would be imposed upon the cells but not prove sufficiently toxic to stop growth completely and/or kill the cells. Two experiments were conducted to assess the dose-response toxicity of the TNT to the cell culture line employed.

In the first experiment, four 100-mL fractions were taken from a 500-mL cell suspension just entering the log phase. A 5-mL aliquot was taken from each of the fractions and assayed for respiration rate (O_2 uptake) with an oxygen electrode (YSI Instruments, Yellow Springs, OH). Three of these were then exposed to either 1, 10, or 100 mg/L TNT for 1 h while the fourth was retained as a control (0 mg/L). Additional 5-mL samples for respiration measurements were then taken immediately following (1 h) exposure. The fractions were then centrifuged at 100 x g and gently washed with fresh medium followed by a final centrifugation and resuspension in 100 mL of the fresh medium. Samples were taken at 24, 48, and 72 h post-exposure and assayed as above. Data [avg. ± s.d. (N=3)], expressed in μMol O_2/h/g dry wt., are given in Figure 3.3.

The results showed that the 1 mg/L TNT did not appear to affect the metabolism of the cultures for up to 72 h post-exposure when compared to the 0 mg/L controls. The 100 mg/L cultures showed a rapid drop in metabolic rate and were dead by 48 h post-exposure. The 10 ppm cultures exhibited an initial decline in respiration rate following exposure when compared to the controls but were apparently recovering and exhibiting growth by 72 h post-exposure.

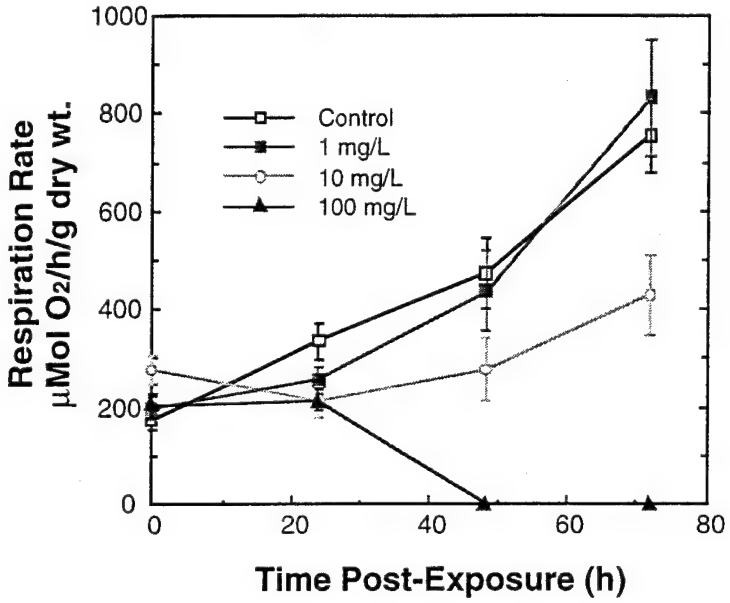

FIGURE 3.3. Respiration Rate (µMol O_2/h/g dry wt.) of Carrot Cell Cultures Exposed to 0 (Control), 1, 10, or 100 mg/L TNT for 1 h

As a followup to determine the effects of long-term exposure, a second experiment was conducted in which the 500-mL cultures were subsampled to make three 100-mL fractions. One of these was then exposed to either distilled water, methanol, or 10 mg/L TNT-methanol for 48 h. After 48 h, the respiration rate was measured as described previously. The results shown in Table 3.4 indicate that no significant effects of long-term (48-h) exposure were evident on the respiration rate of the carrot cells.

These studies indicated that an exposure concentration of 10 mg/L was optimal for our culture and exposure method. This concentration would be expected to stress the isolated cells but not be immediately toxic. If stable and nonlethal genetic mutations can be induced by TNT, then this concentration was anticipated to be a good starting point.

TABLE 3.4 Average Respiration Rates of Carrot Cell Cultures Exposed to 10 mg/L TNT for 48 h. The TNT was dissolved in 0.5 mL of methanol and added to a 100-mL fraction of the cell suspension culture. Data are averages (N=5) ± s.d.

Sample Treatment	Time of Exposure (h)	TNT Concentration (mg/L)	Respiration Rate (μMol O_2/h-g. dry wt.)
Control	48	10 ppm	206±64
Control + Methanol	48	10 ppm	218±39
TNT + Methanol	48	10 ppm	226±25

3.3 MUTATIONAL SPECTRUM STUDY

The criteria required for a true biomarker are that 1) the change in a living organism induced by the stressor be stable, and 2) the change be characteristic for the particular stressor. Both of these prerequisites would also need to be statistically verifiable. For this study, and, given the large genomic size of higher plants, the verification process requires large sampling numbers to distinguish a true biomarker from spontaneous or non related mutations.

The use of a suspension culture of isolated cells as a test medium presents both an answer to part of these requirements as well as a potential problem. Cell cultures can be cycled to provide a large population of individual cells of which the majority are statistically at the same developmental stage. Further in a liquid suspension, all of the cells should recieve a similar dosage of the contaminant, thus eliminating the effects of uneven exposure rates to differing tissues within a whole plant. Unfortunately, even though the suspension culture is homogeneous, resolution of specific genetic changes may be lost in the background noise of the large population following an exposure. Therefore, examining individual cell "subclones" or populations derived from single isolated exposed cells is essential.

The cloning of single isolated higher plant cells is technically challenging. *In vitro* cultured plant cells are reported to require unknown "factors," produced by other cells to initiate and maintain the frequency of cell division among the cultured cells to produce the initial microcallus. These supporting nondividing cells are known as feeder cells (Weber and Lark 1979; Shneyour et al. 1984). Also, the feeder cells must remain physically separate from the dividing cells to ensure the cultures remain genetically pure.

Several approaches are available for employing feeder cells to clone single cells. These generally include the use of a layer of feeder cells overlaid with semi-solid agar on which the single cells are placed. Variations include incorporation of the feeder cells directly into the supportive agar, or the use of a permeable physical barrier (a 160-μ pore diameter polyester mesh, Tetko, Inc.) to separate the cell types still suspended in liquid culture. This latter method was chosen because better contact could be maintained between the cell types given their close proximity and the shared liquid culture media. Further, several cells could be placed on a single membrane which was a critical space consideration given the need of >1500 individual cells from each treatment. This number was chosen to provide a final sample number of at least 100 to 400 separate cloned populations for DNA analysis and was projected based on the assumption of an optimal 5% to 25% survival rate.

The experimental design for the exposure was based on the previous work and included obtaining three separate 100-mL fractions from a 500-mL suspension culture just entering the log phase of growth. This was followed by exposure of the first 100-mL fraction of isolated carrot cells to 10 mg/L TNT (dissolved in 0.5 mL methanol), exposure of the second 100-mL fraction to 0.5 mL of methanol alone, and the third control fraction received 0.5 mL of distilled water alone for 24 h. Following exposure, the cells were washed three times with fresh liquid medium and then centrifuged at 100 x g to pack the cells. The cells were partially resuspended in 5 mL of liquid media and spread onto an agar plate. Individual cells were then picked with a micromanipulator and placed on the mesh floating on the feeder cell layer.

Over the following 6 months, many of the microcalli (~40%) grew to the 16 to 100 cell stage and then went quiescent. A greater number (>45 to 50%) showed no growth at all while a small percentage (<10%) of the cells were lost to contamination. In summary, more than than 98% of the initially isolated cells were unable to survive and produce microcalluses. The remaining calli (<2%) were too few to be statistically significant and the experiment was terminated.

4.0 SUMMARY AND CONCLUSIONS

The objective of this effort was to demonstrate that unique, reproducible genetic mutation spectra can be produced in higher plants in response to exposure to TNT. The approach involved development of a DNA repetitive sequence probe as well as a testing media [a clonal cell line of carrot (*Daucus carota*) suspension cells].

Established techniques for the isolation of higher plant DNA from the chosen carrot tissue were modified through the use of lyophilized tissue and extended CTAB extraction procedures. This was able to maximize DNA yield to greater than 80 to 100 μg/g fresh wt. tissue. Initial probing attempts with commercial probes were marginally acceptable, and so a low-C_0t.library was constructed. From this, three colonies from the low-C_0t DNA library were screened and the DNA inserts sequenced.

An isolated carrot root callus culture was established and the cells tested to define exposure conditions. Under our growth conditions an exposure concentration of 10 mg/L was optimal. This concentration would be expected to stress the isolated cells but not toxic for a minimum of 48 h.

A mutational spectrum study was designed using the suspension cell cultures. Suspension cell cultures present both an answer to part of these requirements as well as a potential problem. Cell cultures can be cycled to provide a large population of individual cells of which the majority are statistically at the same developmental stage. Further, in a liquid suspension, all of the cells should receive a similar dosage. Unfortunately, even though the suspension culture is homogeneous, resolution of specific genetic changes may be lost in the background noise of the large population following an exposure. Therefore, it was essential to examine individual cell "subclones" or populations derived from single isolated exposed cells.

The experimental design included three separate 100-mL fractions exposed to either 10 mg/L TNT (dissolved in 0.5 mL methanol), 0.5 mL of methanol alone, or 0.5 mL of distilled water (controls) for 24 h. Following exposure, the cells were washed, resuspended in a small volume of liquid media, and spread onto an agar plate. Greater than 1500 individual cells were then picked with a micromanipulator and placed on an appropriate growth media.

Over the following 6 months, greater than 98% of the initially isolated cells were unable to survive and produce microcalluses. Many of these (25 to 40%) grew to the 16 to 100 cell stage

and then apppeared to go quiescent. These were too small for DNA extraction and were therefore considered lost. While a small percentage (<10%) were lost to contamination, the majority were lost to unknown causes. The remaining calli were too few to be statistically significant, and the experiment was therefore terminated.

The objectives of the study were partially met in that probes to the carrot clonal suspension were developed and an experimental system was devised and tested. Unfortunately, the statistical significance required to demonstrate unique mutational spectra was not achieved within the time frame of the project and so the experiment was terminated. The concept itself remains to be disproved but the highly risky nature of the experimental approach and the need for large numbers of uniform clones to differentiate true mutations suggest that more direct techniques using whole tissues need to be developed. The latter may prove more cost effective and amenable to field validation.

5.0 LITERATURE CITED

Armour, J. A. L., I. Patel, S. L. Thein, M. F. Fey, and A. J. Jeffreys. 1989. "Analysis of Somatic Mutations at Human Minisatellite Loci in Tumors and Cell Lines." Genomics 4:328-324.

Benzer, S. and E. Freese. 1958. "Induction of Specific Mutations with 5 Bromouracil." Proc. Natl. Acad. Sci. USA 44:112-119.

Bell, R. M. 1992. Higher Plant Accumulation of Organic Pollutants from Soils. EPA/600/R-92/138. U.S. EPA, Corvallis Oregon.

Boltz, E. M., P. Harnett, J. Leary, R. Houghton R. F. Kefford, and M. L. Friedlander. 1990. "Demonstration of Somatic Rearrangements and Genetic Heterogeneity in Human Ovarian Cancer by DNA Fingerprinting." Br. J. Cancer 62:23-27.

Chen, R.-H., V. M. Maher and J. J. McCormick. 1990. "Effect of Excision Repair by Diploid Human Fibroblasts on the Kinds and Locations of Mutations Induced by (±)-7*b*,8*a*-Dihydroxy-9*a*,10*a*-Epoxy-7,8,9,10-Tetrahydrobenzo[*a*]Pyrene in the Coding Region of HPRT Gene." Proc. Natl. Acad. Sci. USA 87:8680-8684.

Dixon, R. A. 1985. "Isolation and Maintenance of Callus and Cell Suspension Cultures." In: Plant Cell Culture - A Practical Approach. R. A. Dixon ed., IRL Press, Washington D. C. pp. 1-20.

Eisenstadt, E., A. J. Warren, A. Porter, D. Atkin and J. H. Miller. 1982. "Carcinogenic Epoxides of Benzo[*a*]Pyrene and Cyclopenta[*cd*] Pyrene Induced Base Substitutions via Specific Transversions." Proc. Natl. Acad. Sci. USA 79:1945-1949.

Honma, M., H. Mizusawa, K. Saski, M. Hayashi, T. Ohno, N. Tanaka, and T. Sofuni. 1994. "Demonstration by DNA Fingerprint Analysis of Genomic Instability in Mouse BALB 3T3 Cells During Cell Transformation." Mutation Res. 304:167-179.

Inoue, M., M. Fujita, C. Azuma, F. Saji, and O. Tanizawa. 1992. "Histogenetic Analysis of Ovarian Germ Cell Tumors by DNA Fingerprints." Cancer Res. 52:6823-6826.

Ledwith, B. J., R. D. Storer, S. Prahalada, S. Manam, K. R. Leander, M. J. VanZwieten, W. W. Nichols, and M. O. Bradley. 1990. "DNA Fingerprint of 7,12-Dimethylbenz[a]anthracene-Induced and Spontaneous CD-1 Mouse Liver Tumors." Cancer Res. 50:5245-5249.

Leung, F. C., D. P. Chandler, X.-Z. Shen, A. E. Jarrell, and R. J. Fellows. 1992. "Use of Repetitive Sequences for the Identification of Species-Specific DNA Fragments in *Fusarium oxysporum*." American Phytopathological Society/Mycological Society of America 1992 Joint Meeting, Portand, Oregon.

Matsumura, Y., and D. Tarin. 1992. "DNA Fingerprinting Survey of Various Human Tumors and Their Metatasies." Cancer Res. 52:2174-2179.

Rogers, S. O., and A. J. Bendich. 1988. "Extraction of DNA from Plant Tissues." Plant Molecular Biology Manual, Kluwer Academic Publishers, Dordrecht, Germany.

Roilides, E., J. E. Gielen, N. Tuteja, A. S. Levine, and K. Dixon. 1988. "Mutation Specificity of Benzo[*a*]Pyrene Diolepoxide in Monkey Cells. Mut. Res. 198:199-206.

Ryon, M. G., B. C. Pal, S. S. Talamage, and R. H. Ross. 1984. Database Assessment of the Health and Environmental Effects of Munition Production Waste Products. Final Report. ORNL-6018. Oak Ridge National Laboratory, Oak Ridge, Tennessee.

Sanger, F., A. R. Coulson, B. G. Barrell, A. J. M. Smith, and B. A. Roe. 1980. "Cloning in Single Stranded Bacteriophage as an Aid to Rapid DNA Sequencing." J. Mol. Biol. 143:161-178.

Small, M. J., and D. H. Rosenblatt. 1974. Munitions Production Products of Potential Concern - Phase II. Technical Report 7404. AD919031. U.S. Army Medical Bioengineering R&D Laboratory. Fort Detrick, Frederick, Maryland.

Spanggord, R. J., W. R. Mabey, T. Mill, T.-W. Chou, J. H. Smith, S. Lee, and D. Roberts. 1983. Environmental Fate Studies on Certain Munitions Wastewater Constituents. Phase IV-Lagoon Model Studies. Final Report Phase IV. DAMD17-78-C-8081. SRI International, Menlo Park, California.

Shneyour, Y., A. Zelcher, S. Izhar, and J. S. Beckmann. 1984. "A Simple Feeder-Layer Technique for the Plating of Plant Cells and Protoplasts at Low Density." Plant Sci. Lett. 33:293-302.

Thein, S. L., A. J. Jeffreys, H. C. Gooi, F. Cotter, J. Flint, N. T. J. O'Connor, D. J. Weatherall and J. S. Wainscoat. 1987. "Detection of Somatic Changes in Human Cancer DNA by DNA Fingerprint Analysis." Br. J. Cancer 55:353-356.

Weber, G. and K. G. Lark. 1979. "An Efficient Plating System for Rapid Isolation of Mutants from Plant Cell Suspensions." Theor. Appl. Genet. 55:81-86.

Yang, J.-L., V. M. Maher, and J. J. McCormick. 1987. "Kinds of Mutations Formed When a Shuttle Vector Containing Adducts of (±)-7b,8a-Dihydroxy-9a,10a-Epoxy-7,8,9,10-Tetrahydrobenzo[a]Pyrene Replicates in Human Cells." Proc. Natl. Acad. Sci. USA 84:3787-3791.